IFN-\(\gamma\) Induces Cell Growth Inhibition by Fas-mediated Apoptosis: Requirement of STAT1 Protein for Up-Regulation of Fas and FasL Expression

Xiulong Xu, Xin-Yuan Fu, Janet Plate, and Anita S-F. Chong

Departments of General Surgery [X. X., A. S-F. C.], Medicine [J. P.], and Immunology/Microbiology [J. P., A. S-F. C.], Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612, and Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023 [X-Y. F.]

ABSTRACT

The mechanism by which IFN-\(\gamma\) inhibits tumor cell growth has not been fully understood. Here we report that IFN-\(\gamma\) up-regulated the expression of Fas and Fas ligand (FasL) on HT29 cells, a human colon adenocarcinoma cell line, and subsequently induced apoptosis of these cells. The kinetics of cell death in IFN-\(\gamma\)-treated HT29 cells paralleled the increase in the levels of Fas and FasL expression. We further show that IFN-\(\gamma\) up-regulated the expression of Fas and FasL in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. Correspondingly, IFN-\(\gamma\) induced cell death in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. IFN-\(\gamma\)-induced cell death was inhibited by caspase-1 inhibitors. Our results suggest that cell growth inhibition by IFN-\(\gamma\) is due to apoptosis mediated by Fas and FasL interaction.

INTRODUCTION

The IFNs were initially recognized by their ability to interfere with viral replication and were later demonstrated to affect many cell functions, such as inhibition of cell growth, induction of cell differentiation, and modulation of the immune response (1, 2). IFNs exert their pleiotropic biological effects by transcriptional regulation of gene expression. The intracellular signaling pathway triggered through IFN receptors has been elucidated recently. It involves the activation and tyrosine phosphorylation of Jak-3 and STAT (3).

Numerous inducible gene products of IFN-\(\gamma\) have been identified, such as the MHC class I and FcyRI (2, 3). Recent studies have demonstrated that IFN-\(\gamma\) is able to stimulate tumor and immune cells to express Fas (4-7), a type I transmembrane protein that triggers apoptosis when stimulated by its ligand or by agonist mAb (7, 8). Fas is constitutively expressed in many types of cells but is most abundant in hepatocytes and in epithelia (5-7, 9-11). The ligand of Fas (FasL) has been identified as a Mr 38,000 type II integral protein, which belongs to the TNF family. In contrast to Fas, FasL is expressed only on activated T cells and natural killer cells as well as in some immune-privileged sites, such as the Sertoli cells of testis and the anterior chamber of cornea (8). Upon binding to FasL, Fas transduces a cell death signal that involves the recruitment of the FADD (Fas-associated protein with death domain) protein to the intracellular death domain of Fas and the activation of caspase-8 and -1, two cysteine proteases belonging to the interleukin-1\(\beta\)-converting enzyme superfamily (8). Activated caspase-8 induces the proteolysis of death substrates, such as laminins, actin, and poly(ADP) ribose polymerase, subsequently leading to the morphological changes in the cell cytoplasm and nuclei as well as the degradation of chromosomal DNA (8).

The mechanism by which IFN-\(\gamma\) induces growth inhibition of tumor cells has not been fully elucidated. Recently, Chin et al. (12) reported that IFN-\(\gamma\) induces the expression of p21\(^{\text{WAF1/CIP1}}\), an inhibitor of cyclin-dependent kinase, suggesting that IFN-\(\gamma\) may arrest cell growth by blocking the cell cycle. Nevertheless, several recent studies have demonstrated that Fas and FasL are expressed at high levels in a variety of human tumors, including gliomas (4, 13), breast cancer (14), prostate cancer (15), and hepatomas (16). In the present study, we report that IFN-\(\gamma\) not only up-regulates the expression of Fas but also FasL in two tumor cell lines, subsequently resulting in apoptosis. We further show that up-regulation of Fas and FasL required the expression and activation of STAT1 protein. Therefore, our data provide a novel explanation for the mechanism of IFN-\(\gamma\)-induced cell growth inhibition.

MATERIALS AND METHODS

Materials. Anti-Fas mAb, CH-11 (IgM), was purchased from United Biomedical, Inc. (Uniondale, NY) and used for Fas-mediated cell death assay. Anti-Fas polyclonal antibody was purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-FasL mAbs, clone 33, was purchased from Transduction Laboratories (Lexington, KY) and used for Western blotting assay. Hygromycin B and G418 were purchased from Sigma Chemical Co. (St. Louis, MO). YVAD-cmk and ZVAD-fmk, two specific inhibitors of caspase-1, were purchased from Calbiochem (San Diego, CA). Both inhibitors were dissolved in DMSO at the stock concentration of 10 mM. RNase A, proteinase K, IFN-\(\gamma\), and TNF-\(\alpha\) were purchased from Boehringer Mannheim (Indianapolis, IN). EGF, PDGF, and FGF were purchased from United Biomedical, Inc. Horseradish peroxidase-conjugated goat anti-mouse IgG- and goat anti-rabbit IgG were purchased from Southern Biotechnology, Inc. (Birmingham, AL). ECL detection solution and nitrocellulose membrane were purchased from Amer sham Corp. (Arlington, IL).

Cells. HT29 cells, a human adenocarcinoma cell line, and Jurkat cells, a human leukemia T cell line, were obtained from American Type Cell Collection. HT29 cells were grown in complete McCoy's medium containing 10% heat-activated FBS. Jurkat cells were grown in complete RPMI 1640 containing 10% FBS. U3A and STAT1-transfected U3A cell lines have been described previously (12, 17, 18). U3A cells were grown in complete DMEM medium with 10% FBS and 250 \(\mu\)g/ml hygromycin B; STAT1-transfected U3A cells were grown in the same medium in the presence of 500 \(\mu\)g/ml G418.

DNA Fragmentation Assay. Cells were grown in six-well plates in the complete medium. Upon 80% confluence, cells were washed twice with HBSS and then maintained in medium containing 0.5% FBS in the presence of various stimuli for 48 h. Cells were washed twice with PBS (pH 7.4) and then lifted off with 2.5 mM EDTA/PBS. Cells were pelleted and resuspended in lysis buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing 0.5% sodium sarkosinate and 0.5 mg/ml protease K. The reaction was maintained at 50°C for 1 h, 5 \(\mu\)l RNase A (1 mg/ml) was added to each sample, and incubation at 50°C was continued for another hour. The reaction was terminated by the addition of 8 \(\mu\)l of sample buffer (10 mM EDTA, 0.1% bromphenol blue, and 25% Ficoll), and 7 \(\mu\)l of 1% low melting agarose were added to each sample, then inactivated at 65°C for 5 min. The samples were then added into dry wells of a 2% agarose gel containing 0.5 \(\mu\)g/ml ethidium bromide. The samples from U3A and STAT1-transfected U3A cells were further extracted with phenol-isopropanol:chloroform (25:24:1), then precipitated with ethanol after protein-
ASE K digestion. The samples were then resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and digested by RNase A (0.5 µg/ml) at 37°C for 30 min. Samples were then separated on a 1.2% agarose gel. Electrophoresis was carried out in TBE buffer (5 mM EDTA, Tris-boric acid) at 90 V for 1-2 h.

Cell Death Assay. HT29, U3A, and STAT1-transfected U3A cells were grown in 12-well plates in the complete medium. Upon 80% confluence, cells were washed twice with HBSS and then incubated in the medium containing 0.5% FBS with various stimuli for 48 h. Cells were washed and lifted off with 2.5 mM EDTA/PBS. Cell death was determined by a trypan blue exclusion assay.

Western Blotting Assay. Cells were lysed in Brij 96 buffer [50 mM Tris-HCl (pH 8.0), 1% Brij 96, 150 mM NaCl, 5 mM EDTA, 1 µg/ml leupeptin and aprotinin each, and 1 mM phenylmethylsulfonyl fluoride]. Nuclear cell lysates were prepared, and protein concentrations were measured by using a Bio-Rad protein assay kit. Twenty-fifty µg of protein per sample were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were then transferred to nitrocellulose membrane; Fas and FasL were detected by Western blot using anti-Fas rabbit serum (Santa Cruz Biotechnology) and FasL mAb (Transduction Laboratories, Lexington, KY), respectively, followed by horseradish peroxidase-conjugated secondary antibodies and ECL. Relative intensities of FasL bands were determined on a Personal Densitometer SI (Molecular Dynamics) using IP Lab Gel software (Signal Analytics Corp.).

Statistical Analysis. All analyses to determine the significant difference between untreated and treated cells were performed by using the Super-ANOVA program for Macintosh (Abacus Concepts, Inc., Berkeley, CA). Significant differences were concluded when P was <0.05 by the ANOVA and a post hoc Tukeys compromise test.

RESULTS

Specific Induction of Fas Expression by IFN-γ. Previous studies using flow cytometric analysis and Northern blot have demonstrated that IFN-γ and TNF-α induces the expression of Fas protein in HT29 cells, a human colon adenocarcinoma cell line (6). Using Western blot analysis, we examined the induction of Fas expression in IFN-γ-treated HT29 cells. In addition, we tested whether growth factors, PDGF, EGF, and FGF, were able to induce Fas expression. As shown in Fig. 1A, IFN-γ at the concentration of 100 units/ml dramatically enhanced the Fas expression; EGF and TNF-α only slightly up-regulated expression of Fas protein, whereas EGF and PDGF had no effect on the expression of Fas. In addition, our Western blot analysis revealed that Fas protein existed in two major forms; this phenomenon has been described previously (7) and is due to the differential glycosylation of Fas proteins. Using anti-Fas mAb (CH-11) as a primary antibody in Western blot analysis, similar results were obtained (data not shown). Additional experiments show that the induction of Fas expression by IFN-γ was both time and dose dependent (Fig. 1A, B and C, upper panel). Fas expression was slightly decreased when IFN-γ was used at high concentrations (>50–100 units/ml), probably due to the increasing cell death as described below.

Specific Induction of FasL Expression by IFN-γ. Regulation of Fas expression by IFN-γ in both malignant and normal cells has been reported previously (4–7); however, little is known about how FasL is regulated. Here we tested whether FasL was regulated by PDGF, EGF, FGF, IFN-γ, and TNF-α. As shown in Fig. 1A (bottom panel), IFN-γ and TNF-α increased the expression of FasL by two to four times as determined by densitometric analysis. In contrast, PDGF, EGF, and FGF had no effect on the expression of FasL. We further demonstrated that IFN-γ induced FasL expression in HT29 cells in a dose- and time-dependent manner (Fig. 1, B and C, bottom panel). We attempted to quantitate FasL expression by using reverse transcription-PCR and flow cytometric analysis; however, FasL was undetectable. This is consistent with previously published data showing that FasL was undetectable (19) or very weakly detectable in HT29 cells (20).

Specificity of IFN-γ-induced Cell Death. Previous studies show that IFN-γ inhibits the growth of HT29 cells, whereas EGF stimulates cell growth (12). However, simultaneous induction of Fas and FasL proteins suggests that IFN-γ may induce growth inhibition via the Fas-mediated apoptosis. We first tested whether IFN-γ treatment could induce the HT29 cell death using a trypan blue exclusion assay. HT29 cells were incubated in the presence of 100 units/ml of IFN-γ for 40 h; dead cells were determined by trypan blue staining. The results in Fig. 1A show that IFN-γ caused the death of 66.7% cells. Induction of cell death by IFN-γ was time and dose dependent (Fig. 2, B and C). TNF-α (1000 units/ml) induced death of 54.3% of cells. As expected, the growth factors EGF, PDGF, and FGF did not induce cell death.

Specificity of IFN-γ-induced TUMOR CELI) APOPTOSIS

Fig. 1. Induction of Fas and FasL expression on HT29 cells by IFN-γ. A, specificity of Fas and FasL induction by IFN-γ and other cytokines. HT29 cells grown in 12-well plates were incubated with growth factors (20 ng each of PDGF, EGF, and FGF), IFN-γ (100 units/ml), or TNF-α (1000 units/ml) for 40 h. Cell lysates were prepared, protein concentrations were normalized, cell lysates (40 µg per sample) were separated on a 10% polyacrylamide gel and then transferred to nitrocellulose membrane. Fas and FasL were detected with anti-Fas rabbit polyclonal antibody (upper panel) and anti-Fasl mAb (lower panel), respectively, followed by horseradish peroxidase-conjugated secondary antibody and ECL. B, dose- (D)- and time (O)-dependent induction of Fas and FasL, expression by IFN-γ are shown. HT29 cells were incubated with various concentrations of IFN-γ for 40 h (B) or 100 units IFN-γ for various lengths of time (O). C, cell lysates were prepared; Fas (upper panel) and FasL (lower panel) expression was examined by Western blotting.

O’Connell et al. (19) reported recently that HT29 cells are resistant to anti-Fas-mediated apoptosis, although these cells express high levels of normal transmembrane Fas protein. In contrast, our results show that anti-Fas mAb induced the death of 25.9% and 37% of HT29 cells when cells were grown in the medium containing 0.5 and 10% FBS, respectively (Fig. 2). In both cases, the induction of cell death was statistically significant (P < 0.05), compared with the corresponding no-antibody controls. We further tested whether cytokine and growth factor pretreatment of HT29 cells could enhance anti-Fas-mediated apoptosis. Consistent with previous observations (21), our results show that both IFN-γ (100 units/ml) and TNF-α (1000 units/ml) significantly increased the anti-Fas-mediated cell death in HT29 cells. In addition, treatment of HT29 cells with low concentrations of IFN-γ (0.1 or 1 unit/ml) induced a marked increase in anti-Fas-mediated cell death (Fig. 2B). Surprisingly, treatment of HT29 cells

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with PDGF and EGF (20 ng/ml) also enhanced anti-Fas mAb-induced death to 40.3 and 75.4%, respectively, whereas anti-Fas mAb alone only induced 25.9% cell death (Fig. 2A), suggesting that PDGF or EGF synergizes with anti-Fas mAb to induce apoptosis. Finally, our results show that IFN-γ (100 units/ml) induced cell death of HT29 in a time-dependent manner (Fig. 2C).

IFN-γ-induced DNA Fragmentation in HT29 Cells. To confirm that trypan blue-stained dead cells in IFN-γ-treated HT29 cells had undergone apoptosis, we performed DNA fragmentation analysis. The results in Fig. 3 (left panel) show that IFN-γ (100 units/ml), but not EGF (20 ng/ml) alone, induced DNA fragmentation in HT29 cells. Treatment of HT29 cells with anti-Fas mAb alone induced moderate DNA fragmentation; pretreatment with IFN-γ followed by anti-Fas mAb did not induce more DNA fragmentation because IFN-γ alone already induced intensive DNA fragmentation. Interestingly, EGF treatment, which by itself did not induce apoptosis, sensitized these cells to anti-Fas-mediated apoptosis. These results are consistent with the data of percentage of cell death as determined in a trypan exclusion assay (Fig. 2A). DNA fragmentation was also observed in a positive control of Jurkat cells incubated with anti-Fas mAb for 20 h (Fig. 3A, Lane 3) but not in Jurkat cells incubated with mouse IgM (Fig. 3A, Lane 2).

We then tested whether DNA fragmentation induced by IFN-γ was dose dependent and whether low concentrations of IFN-γ could sensitize HT29 cells to anti-Fas mAb-induced DNA fragmentation. The results in Fig. 3B (right panel) show that IFN-γ (0.1 and 1 unit/ml) did not induce significant apoptosis in HT29 cells, although IFN-γ at these concentrations induced 35 and 45% cell death in trypan blue exclusion analysis, respectively (Fig. 2B). IFN-γ at the concentrations of 5 and 10 units was able to significantly induce DNA fragmentation in these cells and further enhanced anti-Fas-mediated apoptosis.

IFN-γ Induces the Expression of Fas and FasL in STAT1-transfected U3A Cells but not in STAT1-deficient U3A Cells. Previous studies have demonstrated that IFN-γ inhibits the growth of STAT1-transfected U3A cells but not STAT1-deficient U3A cells (12). We postulated that STAT1 activation may play a critical role in IFN-γ-mediated Fas and FasL up-regulation. To test this idea, STAT1-deficient U3A cells and STAT1-transfected U3A cells were treated with IFN-γ or TNF-α; Fas and FasL expression was analyzed by Western blotting. As shown in Fig. 4, IFN-γ (100 units/ml) did not up-regulate the expression of either Fas or FasL in STAT1-deficient U3A cells; in contrast, IFN-γ significantly induced the expression of both Fas and FasL in STAT1-transfected U3A cells. TNF-α (1000 units/ml) had no effect on the Fas and FasL expression in either cell line. These results suggest that STAT1 protein is required for IFN-γ-mediated up-regulation of Fas and FasL expression.

IFN-γ Induces Apoptosis in STAT1-transfected U3A Cells but not in STAT1-deficient U3A Cells. We further tested whether the up-regulation of Fas and FasL in STAT1-transfected U3A cells resulted in apoptosis. The results in Fig. 5A show that IFN-γ (100 units/ml) did not induce STAT1-deficient U3A cell death but induced death in 87% of STAT1-transfected U3A cells. In contrast, TNF-α (1000 units/ml) induced cell death in both cell lines, although STAT1-transfected U3A cells were more sensitive to TNF-α than STAT1-deficient U3A cells. Consistent with these observations, chromosomal DNA integrity analysis (Fig. 5B) shows that IFN-γ induced DNA fragmentation in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells, whereas TNF-α and anti-Fas mAb (CH-11; 0.5 μg/ml) induced DNA fragmentation in both cell lines. These results

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**Fig. 2. Analysis of IFN-γ-induced cell death.** HT29 cells were grown in 24-well plates in the complete McCoy’s 5 medium containing 10% FBS. Upon 80% confluence, complete medium was replaced by DMEM containing 0.5% FBS. Cells were then incubated in the presence of the indicated stimuli. The percentage of cell death was determined by trypan blue exclusion assay. A, specificity of IFN-γ-induced cell death and enhancement by anti-Fas mAb. HT29 cells were incubated with growth factors (20 ng/ml each of PDGF, EGF, and FGF), IFN-γ (100 units/ml), or TNF-α (1000 units/ml) for 40 h, mouse IgM (○), or anti-Fas mAb (CH-11; 0.5 μg/ml; □) was added to each well. Cells were incubated for another 20 h. Single-cell suspensions were prepared, and cell death was monitored by trypan blue exclusion assay. The data are means of three separate experiments; bars, SD. CTR, control; FBS.

**Fig. 3. IFN-γ-induced DNA fragmentation in HT29 cells.** HT29 cells were preincubated with IFN-γ (100 units/ml) or EGF (20 ng/ml) for 40 h, then mouse IgM or anti-Fas mAb (CH-11) (0.5 μg/ml) was added. After 20 h of incubation, cells were collected and lysed in buffer containing 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% sodium Sarkosinate. Cell lysates were then digested by proteinase K and RNase A (0.5 mg/ml each). DNA integrity was examined by electrophoresis on a 2% gel and stained by ethidium bromide. DNA samples from Jurkat cells treated with mouse IgM (Lane 2) or anti-Fas mAb (Lane 3) were included as controls.

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**Fig. 4.** IFN-γ-induced DNA fragmentation in HT29 cells. HT29 cells were grown in 24-well plates. Cells were then treated with IFN-γ (100 units/ml), or TNF-α (1000 units/ml) for 40 h, mouse IgM (●), or anti-Fas mAb (CH-11; 0.5 μg/ml; □) was added to each well. Cells were incubated for another 20 h (○). Single-cell suspensions were prepared, and cell death was monitored by trypan blue exclusion assay. The results in B and C are means of triplicates from one of three independent experiments; bars, SD.

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**Fig. 5.** Analysis of IFN-γ-induced cell death. HT29 cells were grown in 24-well plates in the complete McCoy’s 5 medium containing 10% FBS. Upon 80% confluence, complete medium was replaced by DMEM containing 0.5% FBS. Cells were then incubated in the presence of the indicated stimuli. The percentage of cell death was determined by trypan blue exclusion assay. A, specificity of IFN-γ-induced cell death and enhancement by anti-Fas mAb. HT29 cells were incubated with growth factors (20 ng/ml each of PDGF, EGF, and FGF), IFN-γ (100 units/ml), or TNF-α (1000 units/ml) for 40 h, mouse IgM (○), or anti-Fas mAb (CH-11; 0.5 μg/ml; □) was added to each well. Cells were incubated for another 20 h. Single-cell suspensions were prepared, and cell death was monitored by trypan blue exclusion assay. The data are means of three separate experiments; bars, SD. CTR, control; FBS.
We show that IFN-γ induces apoptosis in two tumor cell lines, HT29 cells, a human colon adenocarcinoma, and U3A cells, a human myeloblastoma. Our unpublished data show that IFN-γ also induced cell death in A431 cells, a human epidermal carcinoma cell line. These results suggest that IFN-γ-mediated growth inhibition in various types of tumors, especially in the FasL-expressing tumors, is at least in part due to the Fas-mediated apoptosis. Several lines of our experimental evidence support this notion: (a) the kinetics of Fas and FasL expression in IFN-γ-treated cells correlated well with that of cell death; (b) the level of Fas in HT29 cells treated with various doses of IFN-γ correlated with the percentage of cell death and levels of DNA fragmentation; (c) Fas and FasL were up-regulated by IFN-γ in STAT1-transfected U3A cells but not in STAT1-deficient U3A cells. Similarly, IFN-γ induced cell death in former but not in later cell line; (d) the inhibitor of interleukin-1β-converting enzyme blocked IFN-γ-induced cell death. These results collectively suggest that up-regulation of Fas and FasL expression by IFN-γ plays a critical role in IFN-γ-induced apoptosis. STAT1 protein plays a central role in IFN-γ-triggered signal transduction.

**DISCUSSION**

Previous studies have demonstrated that Fas is expressed on HT29 cells (6, 10, 19), and that IFN-γ up-regulates Fas expression as detected by flow cytometric analyses (10) and Northern blot (7). In addition, IFN-γ also increases the expression of Fas in several types of tumor cells, including gliomas (4, 13), breast cancer (14), prostate cancer (15), hepatomas (16), and in normal epithelial cells (5, 6, 9). Consistent with these observations, we confirm that IFN-γ and TNF-α up-regulate Fas expression by Western blot analysis. FasL expression has been detected in a number of tumor cell lines as well as in fresh tumor specimens, including human lung carcinomas (22), astrocytomas (23), hepatomas (24), melanomas (25), and colon adenocarcinomas (19). However, the regulation of FasL expression in tumor cells has not been described previously. Our present studies demonstrate that FasL was also up-regulated by IFN-γ in a dose-dependent and a time-dependent manner.

Up-regulated Fas and FasL proteins in IFN-γ-treated tumor cells may interact with each other to induce apoptosis. Using trypan blue exclusion assay and DNA fragmentation analysis, we show that IFN-γ induced apoptosis in two tumor cell lines, HT29 cells, a human colon...
JAK family tyrosine kinases leads to the translocation of STAT1

U3A and STAT1-transfected U3A cells were left untreated or treated with IFN-γ (10 units/ml) in the presence of ZVAD-fmk (20 μM) or DMSO (final concentration, 1%). HT29 cells were left untreated or treated with IFN-γ (10 units/ml) in the presence of ZVAD-cmk (100 μM) or DMSO (final concentration, 1%). After incubation for 36 h, cells were lifted off with 2.5 mM EDTA/PBS and then stained with trypan blue. Cells were counted, and the percentage of cell death was calculated. The results are means of triplicates from one representative of three experiments with similar results; bars, SD.

Tyrosine phosphorylation of STAT1 proteins by JAK family tyrosine kinases leads to the translocation of STAT1 protein into nucleus and activation of the transcription of genes containing the IFN-stimulated regulatory element (2, 3, 26, 27). The function of STAT1 protein in IFN-γ-induced apoptosis has not yet been established. However, IFN-γ induced the expression of Fas and FasL as well as apoptosis only in STAT1-transfected but not in STAT1-deficient U3A cells, whereas anti-Fas mAb induced apoptosis in both cell lines. These results suggest that STAT1 protein is required for up-regulation of Fas and FasL but may not play a role in Fas-triggered proteolytic pathway. In support of this notion, our unpublished results show that EGF, which activates STAT1 in A431 cells but not in HT29 cells, increased the expression of Fas in a former but not in a later cell line. Cloning and sequence analysis of the Fas gene promoter indicate that IFN-stimulated regulatory element does not exist in this region (28), suggesting that STAT1 may indirectly up-regulate the expression of Fas.

Although our observations suggest that IFN-γ-induced apoptosis is the consequence of interaction of up-regulated Fas and FasL, they do not rule out the possibility of involvement of other mechanisms. The progression of apoptosis is complex, it involves not only the interaction and activation of a cascade of proteases (8) but also several other intracellular proteins such as those of the Bcl-2 superfamily and two tumor suppressor gene products, p53 and IRF-1. A recent report shows that the functional p53 is required for Fas-mediated apoptosis in HepG2 hepatoma cells induced by anticancer drugs, bleomycin and methotrexate (16). IFN-γ does not regulate the expression of p53 but is able to augment the IRF-1 expression (27). Tanaka et al. (29) demonstrated that IRF-1 is required for apoptosis of the dominant Ras-transfected embryonic fibroblast cells induced by blockade of cell proliferation or anticancer drugs. Similarly, Tamura et al. (35) reported that IRF-1 induces the production of Ced-3 protease in activated T lymphocytes and is required for the induction of anticancer drug- and radiation-mediated apoptosis. Therefore, it is possible that enhanced expression of IRF-1 may play a synergistic role in the Fas-mediated apoptosis of IFN-γ-treated HT29 and STAT1-transfected U3A cells. In support of this notion, Keane et al. (14) recently showed that up-regulated IRF-1 by IFN-γ up-regulates the expression of caspase-8, a protease of the caspase family that is involved in the Fas-triggered proteolytic pathway. Up-regulation of caspase-8 results in the sensitization of these tumor cells to anti-Fas-mediated apoptosis. Consistent with these observations, Chin et al. (30) and Kumar et al. (31) more recently reported that the STAT1 signaling pathway is required for the expression of caspase-1 and apoptosis in A431 cells and in STAT1-transfected U3A cells. Taken together, these results suggest that IFN-γ may induce apoptosis of tumor cells by multiple mechanisms.

It is interesting to notice that EGF and PDGF, although unable to increase the expression of Fas and FasL in HT29 cells, augmented anti-Fas-induced cell death and DNA fragmentation (Figs. 2A and 3A). EGF and PDGF bind their corresponding receptors, activate receptor tyrosine kinases, and trigger several signal transduction pathways, such as MAP kinase and JNK pathways. Emerging evidence indicates that MAP kinase and JNK signal transduction pathways cooperate with the Fas-mediated proteolytic pathway (32-34). Recent studies by Goillot et al. (33) have demonstrated that activation of MAP kinase and JNK kinase pathways is required for Fas-mediated apoptosis, and that transfection of dominant-negative mutants of JNK and ERK kinases blocks anti-Fas-induced cell death in SHEP cells, a human neuroblastoma cell line. Therefore, sensitization of HT29 cells to anti-Fas mAb-mediated apoptosis by PDGF and EGF may be due to the synergistic effects between activated MAP/JNK kinase pathways and Fas-triggered proteolytic process.

Earlier studies by O’Connell et al. (19) show that HT29 cells, although expressing high levels of Fas, are resistant to anti-Fas-mediated apoptosis. We have repeated that experiment; however, we found that anti-Fas IgM mAb (0.5 μg/ml) induced significant DNA fragmentation of HT29 cells when grown in the medium containing either 0.5% serum (Fig. 3A, Lane 7) or 10% serum (data not shown). In addition, anti-Fas mAb also induced the death of 35–40% HT29 cells (Fig. 2, A and B). A plausible explanation for this discrepancy is the different concentrations of anti-Fas mAb being used; 0.5 μg/ml of anti-Fas mAb (CH-11) was used in our experiments, whereas only 0.1 μg/ml of same antibody was used in theirs. Our results suggest that Fas expressed on HT29 cells is functional and capable of transducing a death signal and inducing apoptosis.

In conclusion, our present studies demonstrate that IFN-γ not only up-regulates the expression of Fas but also of FasL in two tumor cell lines. Up-regulation and interaction of Fas and FasL result in tumor cell apoptosis, which is prevented by blocking the STAT1-dependent signal transduction pathway or by the caspase inhibitor. Our results suggest that IFN-γ may inhibit cell growth by Fas-mediated apoptosis, thereby providing a cellular explanation for IFN-γ-mediated antitumor activity.

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